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BISMAP 53, 1-94 - 1985
VOLUME 64 - N. 1 - 1985
Pubblicato il 31 Marzo 1985

BOLLETTINO

DELL'ISTITUTO SIEROTERAPICO MILANESE

ARCHIVIO DI MICROBIOLOGIA ED IMMUNOLOGIA

Fondato da S. BELFANTI

RELATION OF SPASTIC AND FLACCID PARALYSIS TO RETROGRADE TRANSPORT OF ^{125}I-TETANUS TOXIN AND ITS ^{125}I-I_{bc} FRAGMENT. MODULATING EFFECT OF F (ab) ANTIBODIES DIRECTED TO SPECIFIC AREAS ON THE TOXIN MOLECULE

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Key words: *tetanus toxin, I_{bc} fragment, retrograde axonal transport, F(ab) antibody fragments*

Summary - The injection into mice of small doses of tetanus toxin induces spastic paralysis as is well-known, whereas large doses of toxin produce flaccid paralysis. The hypothesis has been put forward that the type of symptoms produced may depend on the axonal transport or the lack of axonal transport of the toxin molecule to the central nervous system. In the present paper we show that the lethal flaccid paralysis occurring in mice injected with a very large dose of toxin develops in the absence of any uptake and axonal transport of the toxin molecule. We also confirm that a tetanus toxin-derived fragment, the I_{bc} fragment, which is not transported retrogradely, produces flaccid paralysis. The blockage with the aid of specific antibody F(ab) fragments of the area on the toxin molecule which is involved in binding and axonal transport does prevent the toxin from being transported to the CNS and causes it to produce flaccid paralysis.

Riassunto - L'iniezione nel topo di una piccola dose di tossina tetanica provoca una paralisi spastica come è ben noto, mentre una dose elevata di tossina induce una paralisi flaccida. È stata proposta l'ipotesi che la natura dei sintomi risultanti dall'azione della tossina tetanica potesse dipendere dalla capacità o dall'incapacità della molecola di tossina di venire trasportata per via assonale nel SNC. Nel presente lavoro abbiamo verificato la validità di questa ipotesi. È stato dimostrato che dosi elevate di tossina uccidono il topo con paralisi flaccida in assenza di un trasporto significativo della molecola di tossina verso il SNC. È stato anche verificato che il frammento I_{bc}, provocando la paralisi flaccida, non viene trasportato per via retrograda verso il SNC. Dall'altra parte, il bloccaggio della regione II_c sulla molecola di tossina, la quale è coinvolta nel trasporto retrogrado intraassonale della tossina per mezzo del frammento anticorpale F(ab) diretto contro il frammento II_c si accompagna ad una soppressione dell'*uptake* e del trasporto assonale della molecola. È anche noto che i complessi (Tossina - F(ab)-II_c) esplicano un effetto del tipo tossina botulinica. All'opposto, il bloccaggio della regione I_{bc} sulla molecola di tossina risulta nella formazione di un complesso (Tossina - F(ab)-I_{bc}) che viene trasportato per via retrograda nel SNC e provoca una paralisi spastica.

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Introduction

Tetanus toxin exerts its main pathogenic action by blockage of transmitter release from presynaptic terminals in the central and peripheral nervous systems. However, there is a difference in sensitivity of various synapses to the toxin. In the central nervous system (CNS) the toxin blocks preferentially the inhibitory neurotransmitter release (glycine, GABA) producing a disinhibition of motor neurones which manifests itself as spasticity of corresponding muscles. Later, paroxysmal spasms, convulsions and general spastic paralysis appear and are characteristic features of ascending tetanus. Tetanus toxin injected systemically in mice at high doses blocks the release of excitatory neurotransmitter acetylcholine producing symptoms of flaccid paralysis resembling botulinum poisoning. The toxin reaches the CNS after binding to peripheral motor nerve terminals, retrograde axonal transport and transsynaptic passage to presynaptic nerve endings associated with the perikarya of the neurones innervating the injected region. Apparently the whole toxin molecule is needed to produce the central effect with toxin doses as low as 500 pg per lethal dose, whereas death from flaccid paralysis requires more toxin (250 ng or more) (7).

In a previous study (7) we have shown that the ability of the whole toxin molecule to induce spasticity at low doses and flaccidity at high doses can be altered by antibodies directed to specific parts of the molecule. The modulatory action was obtained by blocking the I_{bc} or the II_c area of the toxin molecule with the corresponding F(ab) antibody fragments. Thus, blockage of the I_{bc} part of the toxin molecule produced a spastic paralysis even at high doses and blockage of the II_c part of the molecule resulted in a flaccid paralysis with low doses of the toxin injected systemically or intracerebrally into mice.

It was postulated that the II_c region of the toxin molecule is essential for binding to nerve terminals, transport to the CNS and action leading to spastic paralysis. When the II_c region of the toxin molecule is blocked, the toxin remains in the periphery and exerts its paralytic action at the neuro-muscular junction. On the other hand, it is known that tetanus toxin fragment I_{bc} and similar fragments B and $\alpha.\beta_2$ produce flaccid paralysis in mice when injected systemically in high doses (7, 10, 12, 13). Antibodies directed to fragment I_{bc} prevent development of the paralysis (7).

In the present study we have compared the neural transport of high or low dosages of ^{125}I-tetanus toxin and toxin fragment ^{125}I-I_{bc} injected intramuscularly (i.m.) in mice and related it to development of spasticity or paralysis To further clarify the relationship between binding and transport of the toxin molecule or lack of it to development of spastic or flaccid paralysis we have also used ^{125}I-tetanus toxin complexed with antibody fragments F(ab) directed to I_{bc} or II_c region of the toxin molecule. The results support our former assumption that transport of tetanus toxin to the CNS is essential for expression of spasticity and that the II_c region must be preserved. I_{bc} region was confirmed to play a role in development of flaccidity and no role in transport to the CNS.

Materials and Methods

Tetanus toxin: It was purified from the filtrate of a 5 day-old culture of *Clostridium tetani* essentially as described elsewhere (1). The purified tetanus toxin preparation contained 3150 flocculation units (Lf) per mg nitrogen and 1.2×10^8 MLD's per mg nitrogen.

Tetanus toxin-derived I_{bc} fragment: It was obtained from papain digested toxin essentially as reported elsewhere (4). On SDS-PAGE, I_{bc} fragment migrated as 2 bands which nearly fused. A molecular weight (M_r) close to 95,000 could be estimated. On immunodiffusion against a complex antitetanus antiserum, I_{bc} yielded only one precipitation line. The precipitation line given by I_{bc} fragment with a complex tetanus antiserum crossed the line given by II_c fragment.

Tetanus toxin-derived II_c fragment: It was prepared by digestion of purified tetanus toxin with papain coupled to CNBr-activated Sepharose 4B (2) (5). On SDS-PAGE, II_c fragment migrated as 2 closely associated bands for which a mean molecular weight of 45.000 could be calculated. On immunodiffusion against a complex tetanus antiserum, II_c fragment gave only one precipitation line showing a cross-reaction with the line of the toxin.

Antisera: These were raised in rabbits against II_c and I_{bc} fragments. The antigens were dissolved in 0.07 M disodium phosphate (pH = 8.2), emulsified with an equal volume of complete Freund's adjuvant (DIFCO: H 37 Ra) and injected into rabbits in the footpads of all four legs (4 times 0.1 ml: 0.5 mg total protein). Two weeks later, 1 mg of the immunoadjuvant P40 (3) was injected intravenously (i.v.). The rabbits were boosted 1 week later by an i.m. injection of a similarly prepared emulsion containing 0.2 mg of the protein. The animals were bled 15 days thereafter. In this way antisera were raised directed to I_{bc}fragment (As-I_{bc}), II_c fragment (As-II_c). The activity of antisera was expressed as the number of flocculating units per ml (Lf/ml).

Antibody F(ab) fragments: These fragments were prepared by papain digestion of IgG using immobilized papain (6). First, the IgG fraction of the various antisera was isolated by adsorption onto a column of Protein A-Sepharose Cl-4B (Pharmacia) (8). The F

(ab) fraction of the various IgG preparations was subsequently treated with papain followed by the filtration of the digested product through a column of Protein A-Sepharose Cl-4B. The purity of F (ab) fragments was determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) which showed a single band with an apparent molecular weight in the range of 50,000 Daltons. It was also verified that F(ab) did not result in the formation of a precipitation line by double gel diffusion against the corresponding antigens. The amount of F(ab) fragments was determined spectrophotometrically at 278 nm using an $E^{1\%}_{1cm} = 15.3$. In a previous study (7) the affinity constants of F(ab)-II_c and F(ab)-I_{bc} were found to be in the range of 10^6 M^{-1} and 10^9 M^{-1}, respectively.

Tetanus toxin complexing with F(ab) fragments: An accurately measured quantity of either cold or labeled toxin, as expressed in MLD (or Lf units), was incubated for 1 hour at 37°C and overnight at 4°C with an amount of the desired F(ab) preparation corresponding to a 5-fold to 10-fold molar excess over the Lf amount of toxin (1 If unit of toxin is equal to 1.38×10^{-11} moles). The formation of a precipitate was not observed in any of these mixtures.

We have previously verified that the reaction of the antibody F(ab) fragments with tetanus toxin was complete under the specified conditions and that only the area of the toxin molecule to which the F(ab) fragment is directed was blocked (7).

Anaesthetic: Surgical anaesthesia in mice was achieved by intraperitoneal (i.p.) injection of 0.08 ml of Imalgen 500 (50 mg/ml of ketamine) (IFF Mérieux, France).

Animals: Male Swiss mice of 28-30 g of body weight obtained from IFFA CREDO (L'Arbresle, France) were used in all experiments. The mice were kept at 20° C and supplied with usual lab chow and water ad libitum.

Labeling of tetanus toxin, I_{bc}-fragment and F(ab)-I_{bc}: The toxin and its I_{bc} fragment, as well as the antibody F(ab)-I_{bc} fragments were iodinated according to the method of Greenwood *et al.* (9). For each preparation 100 μg of protein and 1 mCi of tracer free $Na^{125}I$ (Radiochemical Centre, Amersham) were used. The specific radioactivity of the ^{125}I-labeled toxin was 0.8 μCi/μg, that of I_{bc}-fragment 1.5 μCi/μg and that of F(ab)-I_{bc} 5 μCi/μg.

Injection procedure: Microliter syringe (Hamilton) and 30 gauge needle were used to inject test substances into the right gastrocnemius muscle of mice. Test substances were dissolved in 0.1 M phosphate buffer saline (PBS) pH = 7.35. The volumes of test substance injected were in the range of 10 to 20 μl. Injections were made following recovery from anaesthesia in mice that underwent ligation of the right sciatic nerve, or in normal mice.

Neuronal transport: The neuronal transport of the ^{125}I-labeled substances was determined in normal mice and following single ligation of the right sciatic nerve as previously described by Price *et al.* (14, 15, 16). This procedure is now commonly used to determine neuronal and axonal uptake and transport. At predetermined periods of time after injection or prior to imminent death the animals were anaesthetized with ether, decapitated and exsanguinated. Both sciatic nerves were removed from the level of vertebral column to the gastrocnemius muscle (3 cm). They were rinsed in cold physiological saline, blotted on bibulous paper and placed on a Parafilm sheet. The ligated nerve was subdivided into 3 mm segments. Three segments proximal and distal to ligature and 6 corresponding segments from the left unligated nerve were counted separately for presence of radioactivity in a Gamma counter (LKB1272). The radioactivity was expressed as dpm per segment of the nerve. Comparisons were made between dpm of nerve segments taken from unligated nerves on injected side (experimental side) and uninjected side (control side) and between distal and proximal nerve segments of ligated nerves on the injected side and opposite nerve. Uptake and transport were related to substances tested and the time elapsed between injection and harvesting of nerves. A transport gradient was considered to be present in the distal segments of ligated nerves when the radioactivity was accumulating in nerve segments closest to the ligature. In unligated nerves the distal segments have higher activity which is higher on the injected than control side.

We have expressed our observations on uptake of ^{125}I-tetanus toxin, ^{125}I-I_{bc} and ^{125}I-F(ab)-I_{bc} antibody fragment as percent of injected dpm taken up by the whole left (control) nerve, whole right (experimental) nerve, the proximal and distal parts of the ligated nerves. Where clearly established we have noted presence or absence of a gradient at specific time intervals following injection. The data reported represent the dpm averages of corresponding nerve segments taken from 2-4 animals per substance tested. Animals with short survival time are expected to have a lower count of activity. Therefore, we have not set a limit of significance on the uptake. However, the data are significant in comparison to each other.

Experimental design: In mice we have injected unlabeled tetanus toxin, toxin fragment I_{bc} and F(ab) fragment directed against I_{bc}, [F(ab)-I_{bc}] to test the toxicity of these substances and symptoms produced. The dosages used and results obtained are summarized in Table 1 and form the 1 Experimental Group.

The II Experimental Group of mice were injected with ^{125}I-labeled tetanus toxin, I_{bc} and F(ab)-I_{bc} to test their neural uptake and transport. The dosage injected and results obtained are shown in Table 2.

Table 3 shows the effect of ^{125}I-tetanus toxin complexed with F(ab) antibody fragments directed to I_{bc} or II_c area of the molecule. ^{125}I-tetanus toxin incubated with bovine serum albumin (BSA) was used as control for the method.

Results

Injection of tetanus toxin in mice resulted in expected survival times and symptoms which are directly related to the toxin dosage used (see Table 1) and (7). Similarly the ability of I_{bc} to produce flaccid paralysis and death was confirmed. We have observed no effect on mice injected with F(ab)-I_{bc} alone.

As shown in Table 2 there a significant uptake of ^{125}I-tetanus toxin at both 50 and 1,000 MLD dose in sciatic nerves which is higher on the injected side. A gradient of toxin transport formed in sciatic nerves at the two dosages. However, the gradient was definitely more marked at the lower toxin dose because more time was available for gradient formation in mice injected with 50 MLD (survival time 26 hrs) than in mice injected with 1,000 MLD (survival time close to 14 hrs). Consequently, mice injected with 50 MLD of toxin developed symptoms of local tetanus predominating over some flaccidity present. In contrast, mice injected with 1,000 MLD rapidly developed generalized flaccidity and these died before a sufficient amount of toxin could reach the spinal cord to cause typical signs of tetanus. More striking was the effect of a dose of 100,000 MLD of toxin that killed the animals with generalized flaccidity in such a short time that no toxin uptake could take place. These results clearly show that flaccid paralysis will develop in the absence of any uptake and transport of tetanus toxin, if the amount of toxin is sufficient.

In the group of mice injected with ^{125}I-I_{bc} there was no significant uptake of the toxin fragment despite the animals surviving up to 24 hours when they were sacrificed. There were no symptoms of paralysis since iodination could have delayed development of symptoms as compared with mice injected with cold I_{bc}. Nevertheless, during the 24 hour survival one could expect some uptake of it to occur.

As shown in Table 2, ^{125}I-F(ab)-I_{bc} is not taken up or transported in sciatic nerves of mice.

Table 3 summarizes the results of experiments with ^{125}I-tetanus toxin complexed with F(ab) antibody fragments directed to either I_{bc} or II_c part of the toxin molecule. The data show that a high dose (1000 MLD) of ^{125}I-tetanus toxin incubated with BSA produces pure flaccid paralysis within 12 hours (animal sacrificed near death) with significant uptake of the toxin in whole and ligated nerves on injected side. When the toxin was injected as a complex with F(ab)-I_{bc}, the animals survived for considerably longer time and were sacrificed 27 hours after the complex injection. As expected the thus complexed toxin underwent axonal transport, although to a lower extent than the free toxin. Complexing of the toxin with (F(ab)-II_c did result in blockage of both uptake and axonal transport of the toxin molecule.

Discussion

In a previous paper (7) we have confirmed the report by Laird (11) and Matsuda *et al.* (13) that the injection into mice of large doses of tetanus toxin resulted in a botulinum toxin-like effect with induction of a flaccid paralysis. Matsuda *et al.* ascribed this effect of the toxin to the action of the $\alpha.\beta_2$ toxin fragment consisting of the

TABLE 1 - *Effect of Tetanus Toxin and its I_{bc}-Fragment in mice.*

Experiment No.	Material	I.m. injected dose	Death time	Symptoms
1	Tetanus toxin	50 MLD or 2.5 ng	28	Spastic generalized tetanus
2	Tetanus toxin	100 MLD or 5 ng	20	Spastic generalized tetanus and local paralysis
3	Tetanus toxin	1000 MLD or 50 ng	16	Flaccid paralysis
4	Tetanus toxin	10^5 MLD or 5 μg	4	Flaccid paralysis
5	Tetanus toxin	10^5 MLD or 5μg	10(*)	Flaccid paralysis
6	Fragment I_{bc}	40 μg	30	Flaccid paralysis
7	Fragment I_{bc}	80 μg	24	Flaccid paralysis

(*) mice with ligated nerve

TABLE 2 - *Axonal transport and effect of ^{125}I-tetanus toxin, ^{125}I-I_{bc} and ^{125}I-F(ab)-I_{bc}.*

Material	I.m. injected dose	Time of death (D) or sacrifice (S) hrs	% of injected dpm in sciatic nerve					Symptoms
			normal mice		mice with ligated sciatic nerve			
			E (*)	C (**)	P (°)	D (°°)	C (**)	
^{125}I-Tetanus toxin	50 MLD = 2.5 ng 445 dpm	26 (D)	18 gradient	12	16	24 gradient	5	Spastic paralysis
^{125}I-Tetanus toxin	1000 MLD = 50 ng 8900 dpm	14 (S)	2	1.6	0.8	1.5 gradient	0.6	Generalized flaccid paralysis
^{125}-I-I_{bc}	87.5 μg 9.7×10^6 dpm	24 (S)	——	——	0.02	0.03	0.03	No symptoms at the time of (S)
^{125}I-I_{bc}	175 μg 19.4×10^6dpm	24 (S)	——	——	0.02	0.07	0.02	Same as above
^{125}I-F(ab)-I_{bc}	34 ng 3.7×10^5 dpm	24 (S)	0.05	0.01	0	0.02	0.01	No symptoms

(*) E = experimental side
(**) C = control side
(°) P = proximal segment
(°°) D = distal segment

TABLE 3 - *Uptake and axonal transport of ^{125}I-tetanus toxin and ^{125}I-tetanus toxin complexed with F(ab)-I_{bc} and F(ab)-II_c.*

Material	I.m. injected dose	Time of death (D) or sacrifice (S) hrs	% of injected dpm in sciatic nerve					Symptoms
			normal mice		mice with ligated sciatic nerve			
			E (*)	C (**)	P (°)	D (°°)	C (**)	
^{125}I-tetanus toxin	1000 MLD=50 ng 8900 dpm	12(D)	2	0.3	0.5	1.2	0.2	Flaccid paralysis
^{125}I-tetanus toxin + F(ab)-I_{bc}	1000 MLD=50 ng 17000 dpm	27(S)	0.2	0.1	0.18	0.28 Gradient	0.06	No symptoms at the time of (S)
^{125}I-tetanus toxin + F(ab)-II_c	1000 MLD=50 ng 17000 dpm	27 (S)	0.02	0.01	0	0.04	0.03	No symptoms at the time of (S)

(*) E = experimental side (**) C = control side (°) P = proximal segment (°°) D = distal segment

toxin light chain linked through a disulfide bond to the N-terminal 50 KD fragment of the heavy chain. We also verified that our I_{bc} fragment, which is similar to $\alpha.\beta_2$, produced in mice a lethal flaccid paralysis. We further showed that the blockage of the I_{bc} area on the toxin molecule with F(ab) antibody fragments specifically directed to the I_{bc} fragment caused large doses of the thus complexed toxin to induce spastic paralysis in mice. In contrast, the blockage with the corresponding F(ab) antibody fragments of the II_c area on the toxin molecule, which is involved in uptake and transport of tetanus toxin, made small doses of the thus complexed toxin molecule to produce preferentially flaccid paralysis. Consequently, we proposed that the botulinum toxin-like effect was likely to be related to the lack of uptake and transport of the toxin molecule to the CNS.

In the present study we have again verified that large doses of either unlabeled toxin or I_{bc}-fragment produced in mice a lethal flaccid paralysis (see Table 1). In parallel we have investigated the uptake and axonal transport of both low and high doses of ^{125}I-labeled tetanus toxin. For this we have adapted in the mouse the technique described by Price *et al.* (14, 15, 16) for the ligation of the sciatic nerve in the rat. The use of the mouse in place of the rat for these studies appeared to us important in view of the fact that the rat was found to be insensitive to fragment B which is similar to I_{bc} (10). With the aid of this technique we could verifiy both the uptake and axonal transport of iodinated toxin following the injection of both small and large doses of it (see Table 2). The uptake and transport were found to be inversely related to the dose of toxin injected. The more effective transport of a low dose of toxin as compared to that of a large dose most probably results from the prolonged survival of mice injected with a low dose (see Table 2). Moreover, when a very large dose of ^{125}I-labeled toxin was injected (in the range of 100,000 MLD's) into mice, these died from flaccid paralysis in so short a time as to prevent any uptake of the toxin. At this point we can hypothesize that after injection of a large dose of toxin enough of a fragment similar to I_{bc} might form so as to produce flaccid paralysis. In this respect, it must be recalled that I_{bc} fragment is much less toxic than the toxin. Even though the fragment generated in situ from the toxin is likely to be more toxic than the I_{bc} fragment, it is safe to predict that it should nevertheless be less toxic than the whole toxin. The explanation of the peripheral effect of a large dose of toxin by the release from the toxin of a fragment similar to I_{bc} is further supported by the observation of the lack of uptake ad axonal transport of I_{bc} fragment (see Table 2).

The data summarized in Table 3 show that blocking the II_c area of the toxin molecule with F(ab) antibody fragments directed against II_c fragment prevents uptake and axonal transport of the thus complexed toxin molecule to a significant extent. This could explain why such a complexed toxin molecule produces preferentially flaccid paralysis. In contrast, when the toxin molecule has been complexed with F(ab)-I_{bc}, it is taken up and transported intraaxonally and flaccidity is not observed.

In conclusion, it can be assumed that the type of symptoms produced by tetanus toxin (spasticity or flaccidity) may strongly depend on its being taxen up and transported axonally to the CNS or not.

Acknowledgement: The technical assistance of Ms. M. Lévy and Mr. L. Azeroual is gratefully acknowledged.

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